

Metabolism of Phenol and Hydroquinone to Reactive Products by Macrophage Peroxidase or Purified Prostaglandin H Synthase

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Macrophages, an important cell-type of the bone marrow stroma, are possible targets of benzene toxicity because they contain relatively large amounts of prostaglandin H synthase (PHS), which is capable of metabolizing phenolic compounds to reactive species. PHS also catalyzes the production of prostaglandins, negative regulators of myelopoiesis. Studies indicate that the phenolic metabolites of benzene are oxidized in bone marrow to reactive products via peroxidases. With respect to macrophages, PHS peroxidase is implicated, as *in vivo* benzene-induced myelotoxicity is prevented by low doses of nonsteroidal anti-inflammatory agents, drugs that inhibit PHS. Incubations of either ^{14}C -phenol or ^{14}C -hydroquinone with a lysate of macrophages collected from mouse peritoneum ($> 95\%$ macrophages), resulted in an irreversible binding to protein that was dependent upon H_2O_2 , incubation time, and concentration of radiolabel. Production of protein-bound metabolites from phenol or hydroquinone was inhibited by the peroxidase inhibitor aminotriazole. Protein binding from ^{14}C -phenol also was inhibited by $8\ \mu\text{M}$ hydroquinone, whereas binding from ^{14}C -hydroquinone was stimulated by $5\ \text{mM}$ phenol. The nucleophile cysteine inhibited protein binding of both phenol and hydroquinone and increased the formation of radiolabeled water-soluble metabolites. Similar to the macrophage lysate, purified PHS also catalyzed the conversion of phenol to metabolites that bound to protein and DNA; this activation was both H_2O_2 - and arachidonic acid-dependent. These results indicate a role for macrophage peroxidase, possibly PHS peroxidase, in the conversion of phenol and hydroquinone to reactive metabolites and suggest that the macrophage should be considered when assessing the hematopoietic toxicity of benzene.

Introduction

The mononuclear phagocyte, a cell involved in immunological responses (1,2) and hematopoietic cell regulation (3,4), has been implicated as a target of benzene-induced toxicity (5-7). Lewis et al. demonstrated *in vitro* a selective and pronounced inhibition of macrophage function following the addition of various benzene metabolites to the culture medium (5). Recently, Thomas and Wierda reported that bone marrow-derived macrophages, exposed to the benzene metabolite hydroquinone or its oxidation product 1,4-benzoquinone, secreted less interleukin-1 (6), a monokine capable of regulating the synthesis of several hematopoietic factors (8). In addition, MacEachern et al. reported an activation of resident bone

marrow macrophages in mice receiving benzene or its metabolites phenol and hydroquinone (7).

Phenol, hydroquinone, and catechol are major hepatic metabolites of benzene, and their production appears necessary for benzene-induced myelotoxicity (9,10). These phenolic metabolites can undergo a peroxidase-catalyzed activation to compounds that bind to macromolecules (11-14). Peroxidases catalyze the oxidation of phenolic compounds to quinones (12-14), which have been implicated as mediating the toxic effects of benzene (15). Macrophages possess peroxidatic activity contained, at least in part, by prostaglandin H synthase (PHS), an enzyme with both peroxidase and cyclooxygenase activities (16,17). Other types of macrophage peroxidases may occur; however, since gene expression for myeloperoxidase is lost during macrophage development (18), this peroxidase is probably absent. Since phenol and hydroquinone are good reducing co-substrates for the peroxidase of PHS (19), macrophages have the capacity to oxidize these benzene metabolites to compounds capable of reacting with cellular macromolecules. Indeed, Post et al. have

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demonstrated that the macrophage can metabolize phenol to protein-binding species (20).

The role of PHS in benzene-induced myelotoxicity seems particularly relevant, as benzene administration elevates bone marrow levels of prostaglandin E_2 (21,22), a negative regulator of myelopoiesis (4,23). Nonsteroidal anti-inflammatory drugs, known inhibitors of cyclooxygenase, have been reported not only to inhibit this rise in prostaglandin, but to prevent benzene-induced myelotoxicity as well (21,22). Because nonsteroidal anti-inflammatory drugs inhibit the cyclooxygenase-catalyzed formation of prostaglandin G_2 (PGG_2), treatment with these agents would also eliminate the peroxidase-catalyzed reduction of PGG_2 , thereby avoiding the oxidation of phenolic co-substrates, if present, to reactive compounds.

The above evidence suggests that a) the macrophage may be a target of benzene toxicity, b) PHS may be involved with benzene's myelotoxicity, and c) the peroxidase component of PHS in macrophages may activate benzene metabolites to reactive products. The experiments reported here were designed to investigate the activation of two benzene metabolites, phenol and hydroquinone, by macrophage peroxidase and, in the case of phenol, by PHS, a source of peroxidatic activity in macrophages.

Materials and Methods

Materials

^{14}C -Phenol (110 mCi/mmol) and ^{14}C -hydroquinone (22 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Purified prostaglandin H synthase (PHS; 42,000 units/mg protein) was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Ultra-pure phenol (+99%), hydroquinone, and 1,4-benzoquinone were purchased from Fisher Scientific (Pittsburgh, PA). Protosol and DNA markers were obtained from NEN/Dupont (Boston, MA). Bovine serum albumin (BSA), calf thymus DNA (type 1), Pronase, arachidonic acid, hydrogen peroxide, L-cysteine-HCl, 3-amino-1,2,4-triazole, and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased at the highest available grade of purity.

Preparation of Macrophage Lysate

Macrophages were obtained from the peritoneal cavity of male C57BL/6 mice (7–12 weeks old) 5 days following an IP injection of 1.5 mL of a sterile 10% protease peptone solution. Peritoneal cells were collected by centrifugation, and erythrocytes were lysed with 155 mM NH_4Cl , 0.1 mM EDTA, and 10 mM KHCO_3 . Macrophages were purified by adherence to plastic Petri dishes, which involved incubating peritoneal cells ($2 \times 10^6/\text{mL}$) in RPMI-1640, containing 10% fetal calf serum, for 2 hr at 37°C with 5% CO_2 . Over 95% of adherent cells are macrophages as assessed in this laboratory by morphology,

nonspecific esterase-positive staining, and phagocytosis of sheep red blood cells. Macrophages were scraped from the dishes, and cells ($2 \times 10^6/\text{mL}$) were lysed by homogenization in ice-cold 0.1 M phosphate buffer, pH 7.0, containing 0.1% CTAB. The resulting lysate was centrifuged (1500g, 10 min), and the supernatant was collected and used as the source of macrophage peroxidase.

Activation of ^{14}C -Phenol and ^{14}C -Hydroquinone by Macrophage Peroxidase or Prostaglandin H Synthase

Standard incubation mixtures (complete system) contained 0.5 mL of macrophage lysate (2×10^6 cells lysate/mL), 0.5 mM of either ^{14}C -labeled phenol or hydroquinone (1500 dpm/nmole), 0.5 mM H_2O_2 , and 2 mg/mL BSA diluted to a final volume of 1 mL with 0.1 M phosphate buffer, pH 7.0. Reactions were run at 37°C and were initiated by the addition of H_2O_2 . In some experiments, purified PHS (250 units/mL) replaced the macrophage lysate. These reactions were initiated with either H_2O_2 (0.5 mM) or arachidonic acid (0.15 mM) and were contained in a total volume of 0.5 mL. The cyclooxygenase activity of purified PHS was determined prior to experiments by monitoring oxygen uptake using a Yellow Springs oxygen monitor. Generally, reactions were terminated after 60 min by the addition of trichloroacetic acid (TCA). TCA-precipitable material was washed several times with acetone, hexane:acetone (1:1) and methanol:acetone (1:1). The extracted TCA pellets were solubilized in NaOH and then neutralized with HCl. Radioactivity of the pellets was determined by liquid scintillation spectrophotometry.

For DNA binding studies, incubation mixtures consisted of ^{14}C -phenol, calf thymus DNA (1 mg/mL), and purified PHS (250 units/mL). When DNA was purified from the incubation mixtures, the method of Boyd and Eling (24) was followed, except that RNAase treatment was not employed. The purity of DNA was determined by measuring the A_{260}/A_{280} ratio, which was consistently greater than 1.9. Label irreversibly bound to DNA was determined by pipetting an aliquot onto filter paper discs and washing with TCA, ethanol, and acetone (25). The discs were dried, digested with Protosol, and radioactivity determined by scintillation spectrophotometry.

DNA Binding Assessed with Neutral and Alkaline Gel Electrophoresis

Mitochondrial DNA (mtDNA) was purified from the livers of male Swiss-Webster mice as previously described (26). Aliquots from incubations containing mtDNA (0.1 mg/mL), ^{14}C -phenol (0.5 mM, 5000 dpm/nmole), PHS (250 units/mL), and either arachidonic acid (0.1 mM) or H_2O_2 (0.5 mM) were subjected to electrophoresis following the procedure of Backer and Weinstein (27). Samples (1 μg DNA/well) were loaded onto a 0.7% agarose gel in a Tris (0.09 M)-borate (0.09 M)-EDTA buffer at pH 8.3 and electrophoresed at 25 volts for 16 hr

at 22°C. Gels were stained with ethidium bromide (10 µg/mL) and photographed under ultraviolet light. Gels were then dried and autoradiographed for 5 days at -70°C. In another experiment, mtDNA was electrophoresed in an alkaline gel according to established procedures (28,29). Alkaline gel electrophoresis dissociates double-stranded DNA into unfolded, single strands (28).

Statistical Analyses

Results are expressed as the mean \pm standard deviation of triplicate incubations. Reaction mixtures that contained various treatment regimes were compared to the standard incubation (complete system) with one-way ANOVA followed by Dunnett's *t*-test. (30). The criterion for significance was set at $p \leq 0.01$.

Results

Activation of ^{14}C -Phenol and ^{14}C -Hydroquinone to Protein-Binding Metabolites

Incubations of the macrophage lysate with BSA and ^{14}C -phenol or ^{14}C -hydroquinone resulted in an irreversible binding to TCA-precipitable material that was dependent upon H_2O_2 , incubation time, and the concentration of radiolabeled phenol and hydroquinone (Figs. 1 and 2).

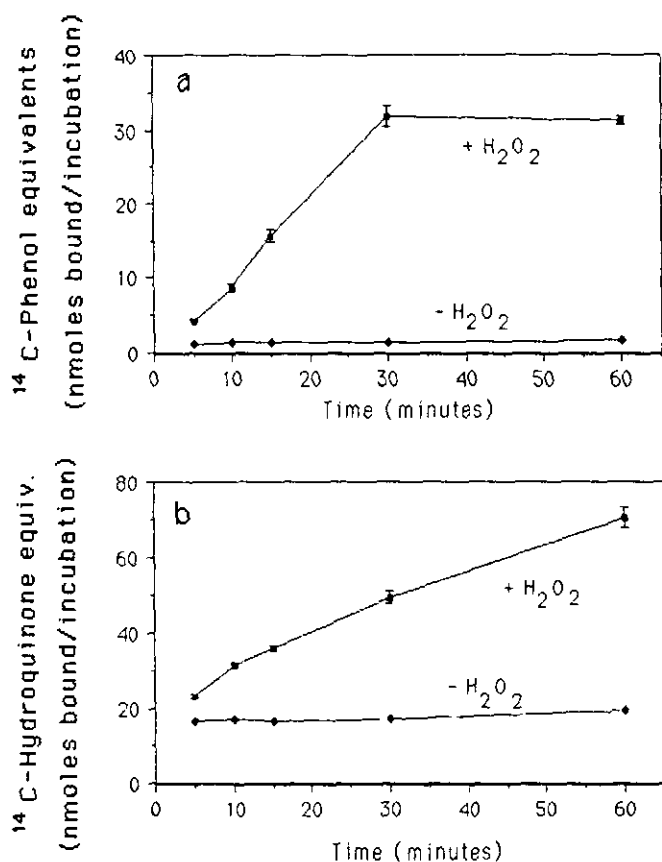


FIGURE 1. The effect of H_2O_2 and incubation time on the activation of ^{14}C -phenol (a) and ^{14}C -hydroquinone (b) to protein-binding metabolites catalyzed by the macrophage lysate.

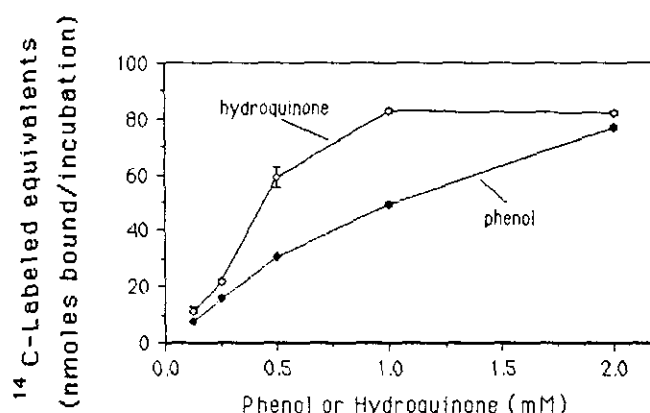


FIGURE 2. The effect of ^{14}C -phenol and ^{14}C -hydroquinone concentration on their conversion to protein-binding metabolites catalyzed by the macrophage lysate. Data points represent the net binding of radiolabeled equivalents (i.e., reactions without H_2O_2 were subtracted from the complete system).

The H_2O_2 -dependent binding of phenol metabolites increased linearly with time up to 30 min, while binding of hydroquinone equivalents was essentially linear over the 60-min incubation period (Fig. 1). When different concentrations of phenol and hydroquinone were incubated with macrophage lysates and BSA, binding increased linearly up to 2 mM for phenol and up to 1 mM for hydroquinone (Fig. 2).

The binding of phenol or hydroquinone equivalents to protein was decreased significantly, compared to the complete system, for incubations containing the peroxidase inhibitor aminotriazole and for reactions carried out in the absence of either the macrophage lysate or H_2O_2 (Fig. 3). The effect of hydroxyl radical scavengers was investigated to determine if the macrophage lysate indirectly activated phenol and hydroquinone through the generation of hydroxyl radicals. The addition of dimethylsulfoxide (DMSO) or mannitol to standard incubation mixtures unexpectedly resulted in a slight but significant increase in both phenol and hydroquinone equivalents bound to TCA-precipitable materials (Fig. 3).

Figure 4 illustrates the effect of the nucleophile cysteine on the conversion of phenol and hydroquinone to protein-binding metabolites and on the formation of ^{14}C -labeled water-soluble metabolites by macrophage lysates. The reduction of protein binding of phenol and hydroquinone equivalents by cysteine was accompanied by an increase in the formation of water-soluble metabolites, indicating that the reduction in protein binding was due, in part, to a shift in the binding of ^{14}C -labeled metabolites to cysteine.

The effect of phenol on the macrophage-dependent metabolism of ^{14}C -hydroquinone and that of hydroquinone on the activation of ^{14}C -phenol are presented in Figure 5. The addition of 5 mM phenol to standard incubation mixtures containing ^{14}C -hydroquinone resulted in a significant increase in hydroquinone equivalents bound to protein (Fig. 5a). Similarly, a significant increase in the formation of 1,4-benzoquinone, the oxidation product of hydroquinone, was seen in the presence of 10 mM phe-

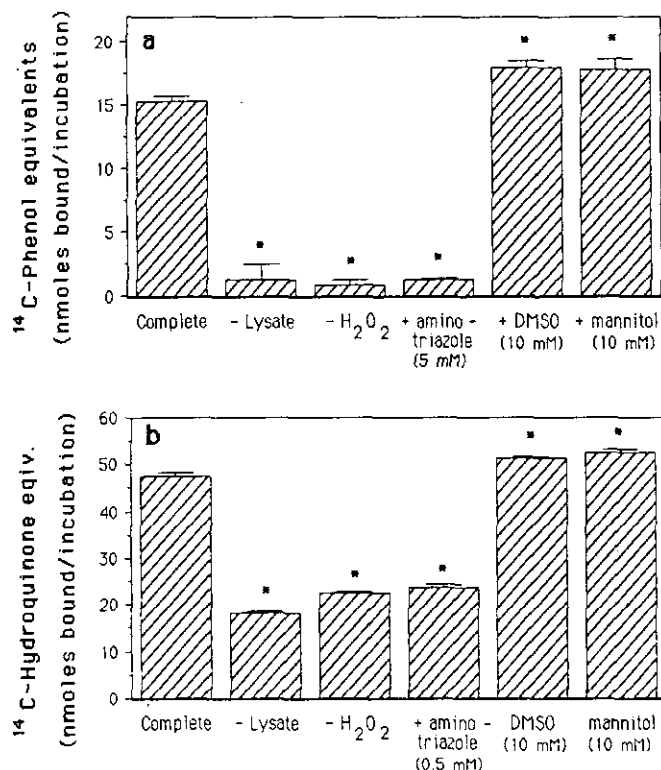


FIGURE 3. The effect of aminotriazole, DMSO, and mannitol on the H_2O_2 -dependent activation of (a) ^{14}C -phenol and (b) ^{14}C -hydroquinone to protein-binding metabolites catalyzed by the macrophage lysate.

nol (Fig. 6). In contrast, the addition of hydroquinone to incubations containing ^{14}C -phenol resulted in a significant decrease in phenol equivalents bound to protein (Fig. 5b).

Prostaglandin H Synthase-Catalyzed Activation of ^{14}C -Phenol

The conversion of phenol to protein-binding metabolites was also catalyzed by purified PHS; this binding was both H_2O_2 - and arachidonic acid-dependent (Fig. 7). ^{14}C -Phenol equivalents bound to TCA-precipitable material, from incubation mixtures containing calf thymus DNA and PHS or PHS only, were dependent on either H_2O_2 or arachidonic acid (Fig. 8). Reaction mixtures that contained DNA, PHS, and either H_2O_2 or arachidonic acid demonstrated an approximate 120% increase in phenol equivalents bound compared to incubation mixtures in which DNA was absent. As expected, arachidonic acid-dependent binding of phenol equivalents to DNA that was extracted and purified from the incubation mixture yielded results similar to those in Figure 8 (10.3 ± 0.6 nmole bound/mL of incubation).

Incubation mixtures of mtDNA with purified PHS, ^{14}C -phenol, and either arachidonic acid or H_2O_2 , generated two discrete mtDNA (ethidium bromide-staining) bands after electrophoresis (Fig. 9). A mtDNA band comigrated with both the 21.8-kb phage DNA standard and the control mtDNA; a smaller nonmigrating band remained at the origin. Autoradiograms of the gels demonstrated that ^{14}C -phenol equivalents were bound to

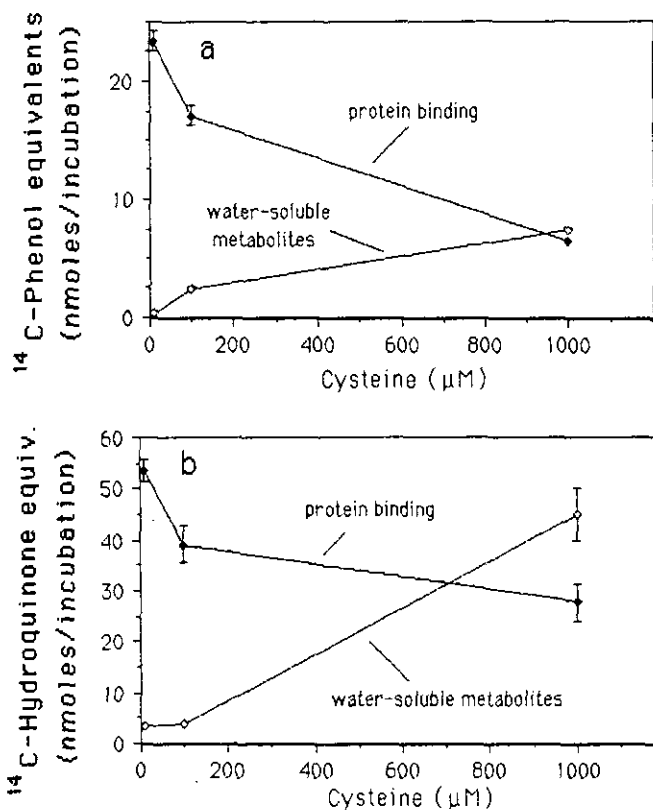


FIGURE 4. The effect of cysteine on the binding of radiolabeled metabolites to protein and on the formation of radiolabeled water-soluble metabolites generated from the macrophage lysate-catalyzed metabolism of (a) ^{14}C -phenol and (b) ^{14}C -hydroquinone. Water-soluble metabolites were obtained from the supernatant of TCA-treated incubation mixtures by extracting four times with an equal volume of ethyl acetate.

mtDNA remaining at the origin (Fig. 10), which suggests that phenol metabolites formed a macromolecular complex with the mtDNA. Similar radioactive patterns were seen for mtDNA electrophoresed in an alkaline denaturing gel (results not shown).

Discussion

This laboratory has previously reported that macrophages convert phenol to protein-binding metabolites (20). In the experiments presented here, binding of phenol and hydroquinone to protein was dependent on the macrophage lysate and H_2O_2 and was prevented by the peroxidase inhibitor aminotriazole. Hydroxyl radical scavengers were studied to determine if the macrophage lysate played an indirect role in protein binding through the generation of hydroxyl radicals, which can occur by either a Fenton or Haber-Weiss reaction. Since hydroxyl radical scavengers did not inhibit the protein binding, but rather had a slight stimulatory effect, our results demonstrated that the activation of phenol and hydroquinone to protein-binding compounds is mediated by a macrophage peroxidase. Previous studies have also demonstrated a peroxidase-catalyzed conversion of phenol and hydroquinone to protein- or DNA-binding compounds;

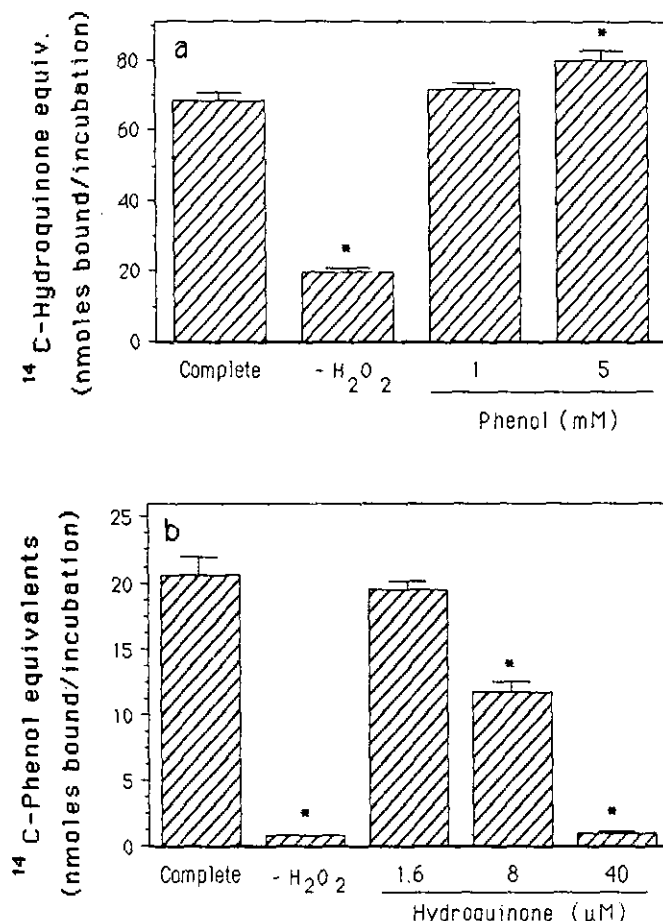


FIGURE 5. Effect of (a) phenol on the conversion of ^{14}C -hydroquinone to protein-binding metabolites and of (b) hydroquinone on the protein-binding of ^{14}C -phenol catalyzed by the macrophage lysate.

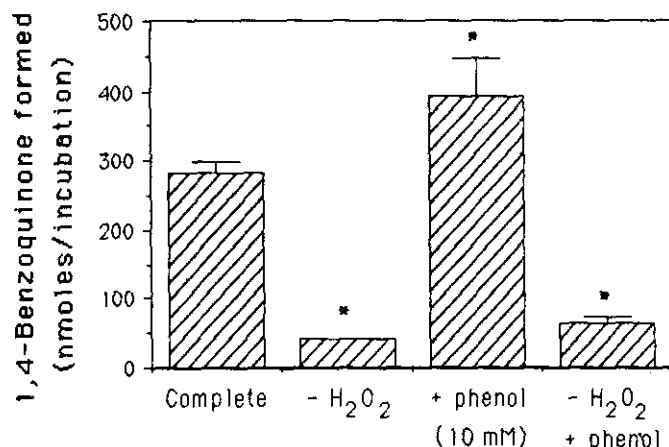


FIGURE 6. The formation 1,4-benzoquinone in the presence or absence of 10 mM phenol. The complete system contained the macrophage lysate (1×10^6 cell lysate/mL), 1 mM hydroquinone, and 0.5 mM H_2O_2 diluted to 1 mL with 0.1 M phosphate buffer, pH 7.0. 1,4-Benzoquinone was assayed using reverse-phase HPLC with electrochemical detection as described previously (31).

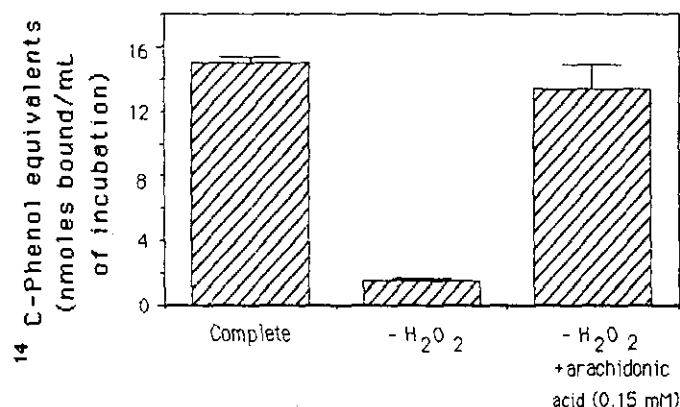


FIGURE 7. The H_2O_2 - and arachidonic acid-dependent metabolism of ^{14}C -phenol to protein-binding species catalyzed by purified PHS.

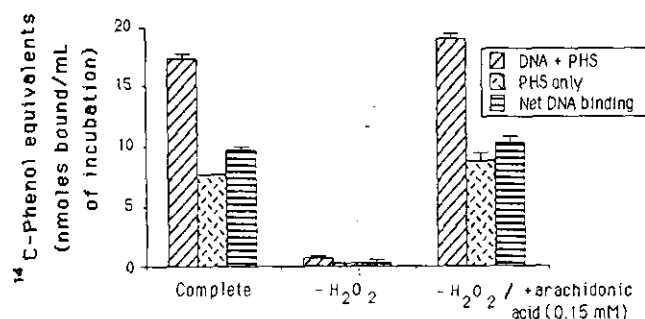


FIGURE 8. The H_2O_2 - and arachidonic acid-dependent conversion of ^{14}C -phenol to metabolites binding to calf thymus DNA and to purified PHS. The net DNA-binding was determined by subtracting the nanomoles bound to the enzyme from the nanomoles bound to both DNA and the enzyme.

however, these investigations used either isolated enzyme preparations or peripheral blood leukocyte peroxidase (11-14).

Cysteine, a nucleophilic amino acid, markedly inhibited the protein binding of phenol and hydroquinone, whereas the formation of water-soluble metabolites increased with the increase in cysteine concentration. These results indicate a reaction of the phenolic reactive metabolites with cysteine. With respect to phenol, about 75% of the reduction in protein binding at 1 mM cysteine could be recovered as water-soluble metabolites, indicating that cysteine-bound metabolites were extracted into ethyl acetate or that cysteine was acting as an antioxidant reducing the phenoxy radical back to phenol. The latter has been demonstrated with another-SH nucleophile, glutathione, which has been shown to reduce phenolic reactive intermediates back to their parent species (32). At 1 mM cysteine, water-soluble metabolites of hydroquinone were dramatically increased, even though not much effect on protein binding was observed, suggesting that cysteine may have bound to metabolites that did not bind protein.

The presence of phenol in reaction mixtures containing



FIGURE 9. A 0.7% agarose gel stained with ethidium bromide and photographed under ultraviolet light. Lane 1, phage-DNA standards; lane 3, mtDNA, ^{14}C -phenol and arachidonic acid; lane 4, mtDNA, PHS, ^{14}C -phenol and arachidonic acid; lane 5, mtDNA, ^{14}C -phenol and H_2O_2 ; lane 6, mtDNA, PHS, ^{14}C -phenol and H_2O_2 ; lane 8, mtDNA only.

hydroquinone, macrophage lysate, and H_2O_2 resulted in a significant increase in the formation of 1,4-benzoquinone, a putative reactive metabolite of benzene (15). The ability of phenol to stimulate the oxidation of hydroquinone, as well as other phenolic compounds, has been previously demonstrated with purified peroxidase enzymes (14,33). These phenolic compounds are thought to be directly oxidized by the phenoxy radical generated from phenol, with this phenol-mediated oxidation occurring in addition to the peroxidase-catalyzed oxidation. Phenol not only stimulated the formation of 1,4-benzoquinone by the macrophage lysate, but significantly

elevated ^{14}C -hydroquinone equivalents bound to protein, which was first demonstrated in a peroxidase system by Eastmond et al. (14). On the other hand, hydroquinone inhibited the macrophage-mediated conversion of ^{14}C -phenol to protein-binding metabolites. This inhibition was expected since hydroquinone and phenol compete as reducing substrates for peroxidases (14,19).

The nature of the macrophage peroxidase is not known; however, the peroxidatic activity of PHS is likely since macrophages have the capacity to secrete relatively large amounts of prostaglandins (16). The peroxidase component of PHS is known to catalyze a variety of compounds

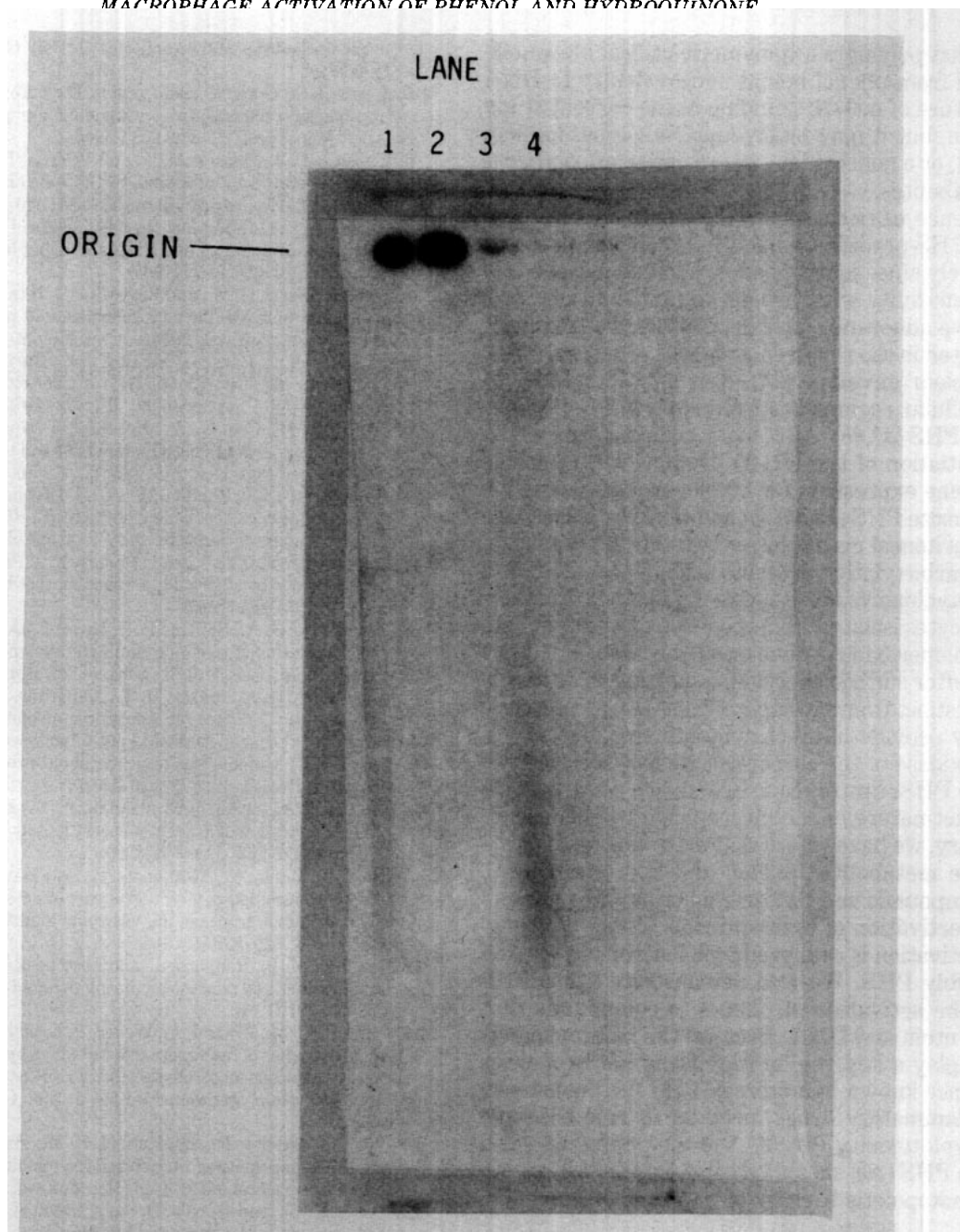


FIGURE 10. Autoradiogram of a 0.7% agarose gel. Lane 1, mtDNA, PHS, ^{14}C -phenol and H_2O_2 ; lane 2, mtDNA, PHS, ^{14}C -phenol and arachidonic acid; lane 3, mtDNA, ^{14}C -phenol and H_2O_2 ; lane 4, mtDNA, ^{14}C -phenol and arachidonic acid.

to reactive intermediates (34). In the current study, the arachidonic acid-dependent activation of hydroquinone and phenol by the macrophage lysate was not attempted, since the concentration of CTAB used to solubilize the membrane-bound peroxidase also inhibits the cyclooxygenase activity of PHS (data not presented). However, the addition of increasing concentrations of arachidonic acid to whole cell macrophages resulted in a linear increase in ^{14}C -phenol equivalents bound to protein (22).

The present study demonstrated a PHS-catalyzed activation of phenol to metabolites that bound to albumin, calf thymus DNA, and mtDNA. The phenol metabolites

bound to mtDNA formed a macromolecular complex that no longer migrated into either a neutral or alkaline gel. Similar electrophoretic patterns of DNA have been described for several carcinogens activated by horseradish peroxidase (35). Alkaline gel electrophoresis would be expected to dissociate double-stranded DNA into single stands migrating into the gel (28). Therefore, oxidized products of phenol that bound to mtDNA probably prevented its separation into distinct single stands. Subrahmanyam and O'Brien examined the nature of the reactive species involved in the horseradish peroxidase-catalyzed binding of ^{14}C -phenol to calf thymus DNA, and

concluded that polymers or polymeric-radical intermediates formed from phenol bound noncovalently to DNA (12). The nature of mtDNA binding in the current study is not known, but it may involve mtDNA cross-linking, aggregation, or a noncovalent intercalation of polymeric phenol metabolites.

The presence of peroxidases (i.e., myeloperoxidase) other than PHS-peroxidase in our macrophage preparation is unlikely since macrophages, elicited into the rabbit peritoneal cavity with various stimuli and then purified by active adherence for 2 hr, contained peroxidatic activity located in the rough endoplasmic reticulum (rER) and perinuclear envelope (36). Peroxidatic activity in these subcellular regions has been reported to be associated with PHS (37,38). Also, macrophages formed during differentiation of the HL-60 promyelocyte cell line lose their gene expression for myeloperoxidase and begin to synthesize PHS (18,39). In addition, the peroxidase of elicited peritoneal macrophages lack the ability to catalyze the decarboxylation of amino acids, which indicates it is not a myeloperoxidase-type enzyme (40). Furthermore, monocytes isolated from the blood of a patient deficient in myeloperoxidase developed peroxidatic activity in the rER after surface adhesion, indicating that this enzyme was distinct from myeloperoxidase (41). Since these studies only provide indirect evidence that the macrophage peroxidase of the current study is associated primarily with PHS, future experiments are needed to define the exact nature of the macrophage peroxidase(s).

In summary, we have shown that macrophages activate the benzene metabolites phenol and hydroquinone to reactive compounds and that phenol stimulates the oxidation and activation of hydroquinone. These data suggest that activation is catalyzed by a macrophage peroxidase, possibly PHS. We also demonstrated that PHS catalyzed the activation of phenol to compounds that bound to protein and DNA. Because the macrophage is thought to play a key role in regulating hematopoiesis (3,4), and since known inhibitors of PHS (i.e., nonsteroidal anti-inflammatory drugs) prevent *in vivo* benzene-induced myelotoxicity (21,22), the macrophage, a cell abundant in PHS (16), should be considered when assessing the hematopoietic toxicity of benzene.

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